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APPLICATION
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TITLE: PEPTIDE-BASED IMMUNOTHERAPEUTIC AGENT
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Specification

PEPTIDE-BASED IMMUNOTHERAPEUTIC AGENTAut
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The present invention relates to a peptide-based immunotherapeutic agent. More specifically, the present invention relates to a peptide-based immunotherapeutic agent useful for an allergy patient who has HLA class II molecules that bind to a specific antigen peptide derived from an allergen, which comprises a specific antigen peptide as an effective ingredient. Furthermore, the present invention relates to a reagent for identifying HLA class II molecules, which comprises an antigen peptide specifically reacting with specific HLA class II molecules.

Background Art

An allergic reaction is an undesirable immunoreaction resulting from the response of an antibody or a sensitized cell to an antigen. An antigen causing an allergic reaction is specifically called an allergen. Allergens include a wide range of substances, such as pollen, mites, animals' epidermis, insects, foods, drugs, and chemicals. An allergic reaction is generally characterized by a two-phase reaction comprising an immediate reaction to an allergen and a subsequent delayed reaction. In the early stage of an allergic reaction, an allergen-specific IgE antibody binds to the surface of basophils in the peripheral blood and mast cells in tissues. When

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an allergen enters the body, the IgE antibodies on the surface of basophils or mast cells cross react with the allergen. As a result, inflammatory mediators including histamine, prostaglandin, and leukotriene are released. In response to these inflammatory mediators, locally accumulated lymphocytes, monocytes, basophils, and eosinophils are activated and release mediators causing tissue damage and other various responses in tissues, thereby initiating a delayed reaction.

It is well known that an allergic reaction is controlled by cytokines. Cytokines are involved in not only the control of IgE production but also the activation and differentiation of effector cells. This is supported by the observation that the level of an allergen-specific IgE in the blood is constant even if clinical symptoms of an allergy patient have been alleviated by hyposensitization.

Sub Hyposensitization, a method for treating allergic diseases, comprises administering a small amount of antigen (for example, an antigen extracted from cryptomeria pollen or mites) to an allergy patient, and increasing the dosage gradually. The success of hyposensitization is attributed to the decreased response of allergen-specific T cells. Presumably, hyposensitization causes T-cell tolerance (T cell anergy), and, as a result, cytokine, which is important for developing an allergic cascade, is not produced. Studies on allergies have focused on the allergen-specific immunoreaction in the early stage, especially on the mechanisms for controlling T-cell response to allergy. An allergic response to an

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C2) exogenous antigen including an allergen is initiated depending on antigen-presenting cells in the immune system. Antigen-presenting cells including B cells, macrophages, and dendritic cells incorporate exogenous antigens, fragment the exogenous antigens into antigen peptides (T-cell epitope peptides), and express the fragmented antigens on the cell surface together with MHC class II (HLA class II for a human) to present an antigen to antigen-specific CD4 positive helper T cells (Th cells).

HLA class II molecules (DR, DQ, and DP) are cell surface antigens composed of α and β chains. The α chain of the DR molecule is encoded by the HLA-DRA gene; the β chain of the DR molecule is encoded by HLA-DRB1, HLA-DRB3, HLA-DRB4, or HLA-DRB5 genes. The α and β chains of the DQ molecule are encoded by HLA-DQA1 and HLA-DQB1 genes, respectively, while α and β chains of the DP molecule are encoded by HLA-DPA1 and HLA-DPB1 genes, respectively. Except for HLA-DRA, each gene comprises numerous alleles. Pockets accommodating antigen peptides composed of α and β chains show high polymorphism, and their structures differ slightly from each other. As a result, kinds of antigen peptides binding to pockets and presented to T cells are limited by their structure. This presumably produces differences in individual immunoreactions.

but C2) Th cells that receive the antigenic information restricted by HLA class II molecules through T-cell receptors (TCR) are activated and secrete various cytokines to proliferate by themselves and differentiate B cells into plasma cells, thereby inducing antibody production. At this time, the second signal (costimulatory signal)

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The decrease of T-cell response to an allergen is related to the success of hyposensitization. For example, T-cell response in vitro to ambrosia allergen "Amb a 1" in a patient suffering from an ambrosia allergy who had undergone effective hyposensitization for ten years was dramatically decreased compared to the untreated patient. Similarly, in a patient allergic to feline epidermis antigen "Fel d 1," T-cell response specific to Fel d 1 was obviously decreased, as hyposensitization showed effects. This decrease corresponded to the decrease of sensitivity in the skin test. Furthermore, IgG and IgE antibodies specific to Fel d 1 remained at a constant level during the treatment. These results indicated that a therapeutic agent for an allergy directly targeting antigen-specific T cells could be prepared.

Development of biochemical separation and analysis techniques has enabled purification of various allergens. In particular, more than 100 kinds of allergen genes have been cloned and their primary structures have been determined in the last several years using techniques in molecular biology and genetic engineering. T-cell epitope sites were also identified in some of those allergens.

Peptide-based immunotherapeutic compositions using peptides including T-cell epitopes of allergens have been disclosed (International patent application published in Japan Nos. Hei 7-

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502890, Hei 8-502163, and Hei 8-507436). When some parts of a T-cell epitope of feline allergen Fel d 1 molecule, but not all of it, were subcutaneously administered to a mouse, antigen-specific T-cell tolerance was reportedly induced against the challenge of whole Fel d 1 (Briner, T. J. et al.: Proc. Natl. Acad. Sci. USA, 90: 7608-7612, 1994). However, whether a major T-cell epitope is effective enough to decrease T-cell response to the challenge of a whole allergen and whether this is can alleviate clinical symptoms have not been confirmed by clinical experiments in humans.

Reportedly, about 3 to 16 T-cell epitope sites exist in an allergen molecule, of which about 1 to 7 sites are recognized by a patient. When the HLA class II type differs in each patient, T-cell epitope sites recognized by each patient also differ. When the HLA class II type is the same, the same T-cell epitope sites are recognized. Thus, the above-described peptide-based immunotherapy using a peptide containing only one major epitope of an allergen molecule recognized in a particular patient population cannot be effective for all patients.

Disclosure of the Invention

An objective of the present invention is to provide a peptide-based immunotherapeutic agent effective for individual allergy patients. Another objective of the present invention is to provide a reagent for typing HLA class II molecules of a patient to be used for selecting a peptide-based immunotherapeutic agent effective for individual allergy patients.

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The present inventors focused on the fact that each patient recognizes different T-cell epitopes of an allergen. The inventors established a method for correlating various T-cell epitopes of allergic molecules with types of the patient's HLA class II molecule that restrict the epitopes. They actually correlated various T-cell epitopes of allergen molecules with types of patient's HLA class II molecules restricting the epitopes in cryptomeria pollen allergens, Cry j 1 and Cry j 2 as follows.

Based on the known HLA-binding motifs of DR, DQ and DP (Rammensee, H. G. et al.: Immunogenet. 41: 178-228, 1995), T-cell epitope sites of allergen molecules can be determined by analyzing the primary structure of allergen molecules and detecting the existence of HLA motifs. Therefore, in order to maximize the possibility that T-cell epitope sites are contained in peptides to be constructed, the epitope sites should be estimated based on known HLA-binding motifs, and peptides should be constructed using said estimated motifs. However, peptides containing estimated HLA motifs do not always cause the expected allergic symptoms. Antigen peptides useful for peptide-based immunotherapy must be determined at least by an experiment using T cells (peripheral blood lymphocytes, T cell lines or T-cell clones). The present inventors identified antigen peptides useful for peptide-based immunotherapy for each HLA type by such an experiment.

The present inventors cultured peripheral lymphocytes, T-cell lines, or T-cell clones derived from a patient sensitive to a specific allergen with antigen-presenting cells and overlapping peptides composed of about 15 to 30 amino acid residues (in which the

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overlapping portion is about 5 to 10 residues) to cover the whole primary structure of the allergen. T-cell response to these peptides was then assayed by measuring the amount of [³H]thymidine uptake (response by cell proliferation). The peptides to which T-cells responded were identified as antigen peptides containing at least one T-cell epitope. Subsequently, the inventors successfully correlated other T-cell epitopes with the patient's HLA class II molecules that restrict the epitopes using various T-cell lines or T cell clones.

Furthermore, the inventors identified T-cell epitopes recognized by a specific mouse. The inventors found that the same mouse which was given said T-cell epitopes exhibited significantly suppressed immune response to the peptides containing said T-cell epitopes. Based on this result, the inventors thought that a peptide-based immunotherapeutic agent effective for individual patients could be provided by selecting T-cell epitope peptides compatible to a type of HLA class II molecule specific to the patient from peptides containing T-cell epitope for which restriction molecules were identified, thereby completing the present invention.

Sub 25 Furthermore, specific T-cell epitopes responding to specific HLA class II molecules can be detected by the method of the present invention. The present inventors considered using the specific T-cell epitopes as a reagent for typing the patient's HLA class II molecules and completed the present invention. The reagent for typing the HLA class II molecules can be effectively used for selecting a peptide-based immunotherapeutic agent effective for individual

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patients.

Specifically, the present invention consists of the inventions described in each claim.

The terms used herein are defined as follows.

"T-cell epitope" means a structure recognized (or responded to) specifically by a T-cell receptor.

~~Def~~ "Recognize" means to activate T cells. Whether T cells are activated or not can be observed by the production of cytokines, such as IL-2, IL-4 and IFN- γ or by DNA synthesis.

"Antigen peptide" means a peptide functioning as an antigen and containing T-cell epitopes.

"Anergy" means a status in which lymphocytes are not activated by antigens and are functionally inactive.

"HLA haplotype" means the combination of HLA class gene loci that are normally inherited as a particular group.

~~Def~~ "Linkage disequilibrium" means the correlation found among different genes when alleles of different HLA loci are recognized in a single chromosome or a haplotype with higher frequency than expected by chance. Linkage disequilibrium is quantified by the difference between the expected and observed values (Δ).

Peptides binding to specific HLA molecules normally contain specific amino acid residues at specific sites. "HLA-binding amino acid motif" means the combination of the sites and kinds of amino acid residues (HLA anchor residues) important for binding to HLA molecules on HLA-binding peptides. Each HLA allele product contains its own motif. An HLA-binding amino acid motif is also simply

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referred to as an HLA-binding motif herein.

See also
In the present invention, T-cell epitopes in an allergen molecule can be mapped by, for example, culturing peripheral blood lymphocytes, T-cell lines, or T-cell clones derived from a patient sensitive to a specific allergen, together with antigen-presenting cells and an overlapping peptide composed of about 15 to 30 amino acid residues (in which the overlapping portion is about 5 to 10 residues) which covers the whole primary structure of said allergen, determining T-cell response to these peptides by measuring the amount of [³H]thymidine uptake (response by cell proliferation), and identifying the peptide to which T-cells responded. The exact epitope sites can be identified by synthesizing deletion variant peptides by subsequently deleting amino or carboxyl terminal amino acid residues of antigen peptides and monitoring the change of T-cell response to these variant peptides. Alternatively, when more than two peptides containing overlapping regions produce T-cell response, the exact epitope sites can be identified by synthesizing new T-cell epitope peptides containing a part or all of the overlapping regions, and monitoring the change of T-cell response. The antigen peptide of the present invention preferably contains at least seven amino acid residues.

T-cell response to antigen peptides can be detected by calculating the stimulation index (SI) which indicates the level of T-cell response to antigen peptides. SI can be calculated by dividing the value (cpm) of [³H]thymidine uptake in response to the peptide by the value (cpm) obtained by using the medium without the peptide.

The SI of an antigen peptide useful for peptide-based immunotherapy of the invention is at least 2.0, preferably at least 2.5, more preferably at least 3.5, and most preferably, at least 5.0.

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An antigen peptide of the present invention has in vitro proliferation activity on peripheral blood lymphocytes, T-cell lines or T-cell clones derived from an individual allergy patient having HLA class II molecules restricting said peptide. An antigen peptide of the invention does not react with an IgE antibody of a patient sensitive to the allergen from which said peptide is derived. The antigen peptide of the present invention can induce antigen-specific T-cell anergy by the administration of the antigen peptide and thereafter can induce immunological tolerance at any time when challenge of a recombinant or natural allergen derived from said antigenic peptide is made. Furthermore, once an antigen peptide of the present invention is administered to an individual sensitized by an allergen, thereafter immunological tolerance can be induced at any time when the challenge of said allergen is made. These facts indicate that the antigenic peptide of the present invention induces an antigen-specific immunological tolerance in vitro and is useful for peptide-based immunotherapy of an allergy patient.

HLA class II molecules of an allergy patient which bind to the antigen peptides can be typed as follows. Identified antigen peptides, self-derived EB lines treated with mitomycin C (B-cell strains transformed by Epstein-Barr virus), and T cells are cultured with anti-HLA-DR antibody, anti-HLA-DQ antibody, or anti-HLA-DP antibody to assay the inhibition of T-cell proliferation response,

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thereby identifying which of molecules DR, DQ or DP restricts the antigen peptide. When identified restriction molecules are either DQ or DP, the type of restriction molecule (DQ or DP) can be identified using EB lines with a known HLA haplotype as antigen-presenting cells (Hori, T. et al.: Tissue Antigen 47: 485-491, 1996). HLA class II DNA typing is performed by extracting DNA from B-cell lines and subjecting it to the PCR-SSO method adopted in the 11th International Major Histocompatibility Conference [Tsuji, K., Aizawa, M. & Sashazwuki, T eds, (1982) HLA-1991 vol.1 pp395-518]. Restriction molecule DR cannot be identified by using EB lines as antigen-presenting cells due to linkage disequilibrium between DRB1* and DR super types (DRB3*, DRB4*, and DRB5*). Therefore, restriction molecules are identified by transforming mouse L-cell with DRB1* or only one type of DR super type and using the transformant expressing the introduced gene as antigen-presenting cells.

Examples of antigen peptides and the HLA class II molecules restricting them are as follows.

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C10* At present, major allergens of cryptomeria pollen allergen, Cry j 1 and Cry j 2, have been isolated and purified. cDNAs of both allergens have been isolated, and their estimated primary structures have been disclosed (International patent application published in Japan Nos. Hei 8-502163 and Hei 8-505284). T-cell epitope sites in the Cry j 1 molecule were identified based on the molecules primary structure. A therapeutic composition for cryptomeria pollen allergy, composed of a peptide containing the epitope site as an effective ingredient, has been disclosed (International patent application

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- 1) DRB5*0101 of a patient suffering from a cryptomeria pollen allergy, binds to antigen peptides p106-120 (SEQ ID NO: 3) and p109-117 (SEQ ID NO: 4) derived from Cry j 1, and antigen peptides p66-80 (SEQ ID NO: 14) and 236-250 (SEQ ID NO: 19) derived from Cry j 2,
- 2) DRB4*0101 binds to antigen peptides p191-205 (SEQ ID NO: 7) derived from Cry j 1 and antigen peptides p186-200 (SEQ ID NO: 18) derived from Cry j 2,
- 3) DQA1*0102-DQB1*0602 binds to antigen peptides p16-30 (SEQ ID NO: 1), p146-160 (SEQ ID NO: 5), p191-205 (SEQ ID NO: 7), p251-265 (SEQ ID NO: 9), and p326-340 (SEQ ID NO: 10) derived from Cry j 1 and antigen peptides p326-340 (SEQ ID NO: 21) and p341-355 (SEQ ID NO: 23) derived from Cry j 2,
- 4) DPA1*0101-DPB1*0501 binds to antigen peptides p61-75 (SEQ ID NO: 2) and 221-225 (SEQ ID NO: 8) derived from Cry j 1 and antigen peptide p76-90 (SEQ ID NO: 15) derived from Cry j 2,
- 5) DPA1*0202-DPB1*0501 binds to antigen peptide p336-350 (SEQ ID NO: 22) derived from Cry j 2,
- 6) DPA1*0101-DPB*201 binds to p181-195 (SEQ ID NO: 17) derived from Cry j 2,
- 7) DRB1*0901 binds to antigen peptides p151-165 (SEQ ID NO: 6) and p191-205 (SEQ ID NO: 7) derived from Cry j 1, and antigen peptides 16-30 (SEQ ID NO: 12) p151-165 (SEQ ID NO: 16) and p321-335 (SEQ ID NO: 20) derived from Cry j 2, and
- 8) DRB1*1501 binds to antigen peptides p36-50 (SEQ ID NO: 13) and p236-250 (SEQ ID NO: 19) derived from Cry j 2.

A core sequence of antigen peptide p106-120 (SEQ ID NO: 3) derived from Cry j 1 is p109-117 (SEQ ID NO: 4).

Ikagawa et al. reported that Cry j 1 antigen peptide p335-346 (SEQ ID NO: 11) was presented by DRB3*0301 (Ikagawa, S. et al.: J. Allergy Clin. Immunol. 97: 53-64, 1996). Hori et al. reported that Cry j 1 antigen peptide p214-222 (SEQ ID NO: 24) was presented by DPA1*0202-DPB1*0501 (Hori et al.: Tissue Antigens, 47: 481-491, 1996).

It has been conventionally hypothesized that there is a bias for restriction molecules in the level of HLA class II locus depending on the types of antigens. The above studies revealed that, in principle, all DR, DQ, and DP molecules are used as restriction molecules presenting antigen peptides derived from Cry j 1 or Cry j 2, without bias.

Major HLA class II molecules binding to the specific antigens in Cry j 1 include DPA1*0101-DPB1*0501 binding to p61-75 (SEQ ID NO: 2), DQA1*0102-DQB1*0602 binding to p146-160 (SEQ ID NO: 5), and DPA1*0101-DPB1*0501 binding to p211-225 (SEQ ID NO: 8). In Cry j 2 these include DRB1*0901 binding to p16-30 (SEQ ID NO: 12), DRB1*1501 binding to p36-50 (SEQ ID NO: 13), DPA1*0101-DPB1*0501 binding to p76-90 (SEQ ID NO: 15), DRB4*0101 binding to p186-200 (SEQ ID NO: 18), and DPA1*0202-DPB1*0501 binding to p336-350 (SEQ ID NO: 22). The other peptides can bind not only to the identified restriction molecules but also to other molecules, and are thus characterized as multibinder peptides.

In general, an antigen peptide binding to a specific HLA

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molecule contains a common HLA binding amino acid motif. HLA binding amino acid motifs are necessary for antigen peptides to bind to HLA molecules. HLA molecules do not have high selectivity for binding to antigen peptides though other peptide hormone receptors have high selectivity to their ligands. Therefore, HLA molecules can bind to various potential antigen peptides. In HLA class II molecules, a binding motif of an antigen peptide consists of 3 to 5 amino acid residues separately located with 1 to 2 amino acids interposed (Matsushita, S. et al.: J. Exp. Med. 180: 873-883, 1994; Rammensee, H. -G. et al.: Immunogenet. 41: 178-228, 1995). Using these known HLA class II binding motifs, antigen peptides which can bind to the exemplified HLA class II molecules can be further selected based on the primary structure of the cryptomeria pollen allergenic molecule. Therefore, antigen peptides of the present invention which bind to a specific HLA class II type possessed by a patient suffering from a cryptomeria pollen allergy are not limited to antigen peptides exemplified in the present invention, but include antigen peptides expected to bind to specific HLA class II types.

The above-described antigen peptides binding to specific HLA class II types can be used as a peptide-based immunotherapeutic agent for a patient having said HLA class II types. When the antigen peptide of the present invention is used as a peptide-based immunotherapeutic agent to treat an allergy patient, it can be combined with pharmaceutically acceptable diluents and carriers. The resulting composition can be administered by simple methods such as injection (subcutaneous or intradermally), instillation, rhinenchysis, oral

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administration, inhalation, percutaneous application, or trans-mucus. Dosage can be determined by usual methods by one skilled in the art.

To select a peptide-based immunotherapeutic agent suitable for each patient, the HLA class II type of the patient should be determined. The HLA class II type of a patient can be determined by using an antigen peptide specifically reacting with the HLA class II molecule as a reagent.

Specifically, HLA class II molecules of an allergic patient and a healthy subject can be typed as follows. Amino acid motifs of antigen peptides binding to each molecule vary depending on the HLA class II types due to their high polymorphism. HLA class II molecules of a patient and a healthy person can thus be typed by labeling antigen peptides with different binding motifs and detecting the specific binding to HLA class II molecules. Antigen peptides can be labeled by binding a known label, such as a radioisotope, an enzyme, a fluorescent label, or a luminescent label, to an amino acid residue (for example a tyrosine residue) other than the HLA anchor amino acid residues of the antigen peptides. Alternatively, biotinylated antigen peptides are detected with streptoavidin (or avidin) bound to the above label. An allergic patient can be diagnosed by culturing peripheral blood lymphocytes of the subject in the presence of various antigen peptides derived from the allergen and monitoring T-cell response by, for example, adding [³H]thymidine to the culture medium and measuring the amount of [³H]thymidine uptake. Moreover, if T-cell response can be found in a subject (an allergic-response-positive

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C18 patient), the type of the subject's HLA class II molecules restricting the antigen peptides that induced the T-cell response can be identified as the subject's type of HLA class II susceptible to said allergen.

4819 The correlation between the patient's HLA class II type and the antigen peptides identified by this method can be used to study the roll of each HLA class II type in the onset of allergy or to select antigen peptides to be used in a peptide-based immunotherapeutic agent for the allergic patient.

4820 A peptide-based immunotherapeutic agent for a particular allergic patient whose HLA class II molecule type has been identified can be prepared by selecting an antigen peptide compatible with the HLA type of said patient, measuring the response to the peptide to proliferate peripheral blood lymphocytes derived from the patient, and comparing the level of the response of the peptide. For example, the haplotypes of HLA class I and class II of patient PB suffering from cryptomeria pollen allergy described in Example 6 are: A2/24-B39/55-Cw7/w3-DRB1*1501/0901-DRB4*0101-DRB5*0101, and DQA1*0102/0301-DQB1*0602/0303-DPB1*0101/0101-DPB1*0501/0201. When antigen peptides to be used for peptide-based immunotherapy for said patient are selected, the antigen peptides p211-225 (SEQ ID NO: 8) presented by DPA1*0101-DPB1*0501, p106-120 (SEQ ID NO: 3) presented by DBR5*0101, p191-205 (SEQ ID NO: 7) or p251-265 (SEQ IN NO: 9) presented by DQA1*0102-DQB1*0602 should be selected in Cry j 1; in Cry j 2, p76-90 (SEQ ID NO: 15) presented by DPA1*0101-DPB1*0501, p186-200 (SEQ ID NO: 18) presented by DRB4*0101, and p66-80 (SEQ ID

NO: 14) presented by DRB5*0101 should be selected. Before peptide-based immunotherapy is effected using these antigen peptides, the response to these antigen peptides to proliferate peripheral blood lymphocytes derived from the patient should be measured to select the antigen peptides exhibiting a relatively high proliferation activity, which antigen is to be used for peptide-based immunotherapy.

In order to improve solubility, therapeutic or prophylactic effects, and stability of the effects, the antigen peptide of the present invention can be modified by substituting, deleting, or adding amino acid residues other than the HLA anchors without spoiling their function. A certain amino acid can be suitably substituted with Ala, Ser, Glu, or methyl amino acids, but substituent amino acids are not limited thereto. Cys residue forms a dimer through a disulfide bond and functions as a multi-binder. Therefore, immunization with a peptide containing a Cys residue may cause recognition of sites which are not originally involved in antigenicity and thereby create new epitopes. In this case, a Cys residue can be substituted with Ala, Ser, Thr, Leu, or Glu. It may also be substituted by a D amino acid or a non-natural amino acid. A vector capable of expression as a polypeptide, a peptide with a histidine polymer (for example, a histidine hexamer) at its N- or C-terminus, has been developed. The expression product can be purified by affinity chromatography using a nickel chelating column even in the presence of a denaturant. Such an embodiment is also included in the present invention.

The antigen peptide of the present invention can be derived from one allergen molecule or two or more different molecules. All

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protein allergens can be used in the present invention, including pollens of herbage such as ragweed, dactylis, and perennial ryegrass; pollens of arbors such as cryptomeria, chamaecyparis, and mountain cedar; mites; animals; fungi; insects; and foods.

Brief Description of the Drawings

Figure 1 shows the overlapping peptides between Cry j 1 and the patient's T-cell epitope sites. In the figure, \square indicates $2 \leq SI < 5$ and \blacksquare $5 \leq SI$. T-cell clones were prepared from PB and PJ.

Figure 2 shows the overlapping peptides between Cry j 2 and the patient's T-cell epitope sites. In the figure, \square indicates $2 \leq SI < 5$ and \blacksquare $5 \leq SI$. T-cell clones were prepared from PB, PC and PR.

Figure 3 shows the epitope sites recognized by T-cell clones which recognize Cry j 1, the molecules restricting said clones, the production of lymphokines by said clones, and Th types of said clones. In the figure, Th2 stands for $IL-4/IFN\gamma > 10$, Th1 for $IFN\gamma/IL-4 > 10$, and Th0 for the intermediate therebetween.

Figure 4 shows the epitope sites recognized by T-cell clones which recognize Cry j 1, the restriction molecules of said clones, the production of lymphokines by said clones, and Th types of said clones. In the figure, Th2 stands for $IL-4/IFN\gamma > 10$, Th1 for $IFN\gamma/IL-4 > 10$, Th0 for the intermediate therebetween, and Thp for no lymphokine production.

Figure 5 shows immune response of CB6F1 mouse to Cry j 2 when the antigen peptide p66-80 of Cry j 2 was administered to the mouse.

Best Figure 6 shows the immune response of CB6F1 mouse to Cry j 2 when the antigen peptide p186-200 of Cry j 2 was administered to the mouse.

Best Mode for Implementing the Invention

The present invention is illustrated with reference to the following examples, but is not construed to be limited thereto.

Example 1 Purification of cryptomeria pollen antigen

Cry j 1 was purified by the method of Yasueda et al. (Yasueda, H. et al., J. Allergy Clin. Immunol. (1983) 71: 77-86). Cry j 2 was purified by inserting the Cry j 2 gene (unexamined published Japanese patent application (JP-A) No. Hei 8-47392) into expression vector pQE9 (Qiagen GmbH, Germany), transforming E. coli with the vector to express the gene, and purifying the gene expression product by affinity column chromatography using Ni²⁺-NTA-agarose (Quiagen, Inc. USA) (Komiyama, N. et al., Biochim. Biophys. Res. Commun. (1994) 201: 1021-1028).

Example 2 Synthesis of overlapping peptides

Based on the primary structures of Cry j 1 (WO94/01560) and Cry j 2 (JP-A No. Hei 8-47392), 69 kinds of overlapping peptides for Cry j 1 (Figure 1) and 74 kinds for Cry j 2 (Figure 2) were synthesized with a peptide synthesizer (Shimadzu, PSSM-8 model). These overlapping peptides cover all the primary structures of Cry j 1 or Cry j 2 and consist of 15 amino acid residues in which the overlapping portion has 10 residues. The peptides were dissolved in PBS containing 8M urea to 2mM. When the peptides were added to the culture

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system for assaying T-cell proliferation response, the peptides were diluted 500-fold to eliminate the effect of urea.

Example 3 Establishing antigen presenting cells

Peripheral blood lymphocytes were isolated from peripheral blood from a patient suffering from cryptomeria pollen allergy by the Ficoll-paque (Pharmacia) specific gravity centrifugation method. B95-8 cell (derived from a marmoset, ATCC CRL1612) culture supernatant with Epstein-Barr (EB) virus was added to 1×10^6 peripheral blood lymphocytes to infect B cells in peripheral blood lymphocytes with EB virus. Infected B-cells were cultured in RPMI-1640 medium with 200 ng/ml of cyclosporin A and 20% fetal calf serum (FCS) for about 20 days to establish transformant B cell lines.

Example 4 Establishing T-cell lines and T-cell clones

4×10^6 peripheral blood lymphocytes were suspended in 2 ml of RPMI-1640 medium supplemented with 20% human serum, and cultured for 8 days in the presence of 50 μ g/ml of Cry j 1 or 2 to 10 μ g/ml of Cry j 2 to activate T cells recognizing Cry j 1 or Cry j 2.

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C28 When the activated T cells appeared, T-cell lines specifically recognizing Cry j 1 or Cry j 2 were established by replacing the medium with RPMI-1640 medium with 200 U/ml of IL-2 (Boehringer-Mannheim) and 15% human serum and culturing the cells for an additional 14 days.

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C28 T-cell clones specifically recognizing Cry j 1 or Cry j 2 were established as follows. When the activated T cells appeared, T cells were spread in a 10-cm culture dish and selected one-by-one using a micropipet. Separately, the same nonactivated cells transfected with EB virus were treated with mitomycin C (Kyowa Hakko Kogyo) and

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inoculated into each well of a 96-well microculture plate to 1×10^5 cells/well. The above activated T cells were transferred to the 96-well plate, one cell per well. An additional 50 $\mu\text{g/ml}$ of Cry j 1 or 2 to 10 $\mu\text{g/ml}$ of Cry j 2 was added to each well and cultured for 7 days for challenge. The challenge at an interval of 7 days was repeated two or three times to establish T-cell clones.

Example 5 Identifying Cry j 1 and Cry j 2 T-cell epitopes

Peripheral blood lymphocytes derived from 18 patients suffering from cryptomeria pollen allergy were challenged by Cry j 1 or Cry j 2 to establish T-cell lines specifically recognizing Cry j 1 or Cry j 2 for each individual patient. 5×10^4 cells of self-derived B-cell line treated with mitomycin C, $2 \mu\text{M}$ of overlapping peptides, and 2×10^4 cells of the T-cell line were cultured in RPMI-1640 medium supplemented with 0.2 ml of 15% serum in a 96-well microplate for two days. $0.5 \mu\text{Ci}$ of [^3H]thymidine was added, and the culture medium was cultured for an additional 18 hours. The cells were collected in a glass filter with a cell harvester, and uptake of [^3H]thymidine was measured with a liquid scintillation counter. T cells capable of recognizing antigenic information of Cry j 1 or Cry j 2 as well as HLA class II molecules proliferate and took [^3H]thymidine into the cells. Cells exhibiting a Stimulation Index of 2 or higher were recognized as added antigen peptides.

~~291~~ In T-cell epitope sites identified using T-cell lines recognizing Cry j 1, the number of T-cell epitope sites recognized by each patient was 9.8 ± 3.0 on average and ranged from $4 \leq 15$ epitopes. Using T-cell lines recognizing Cry j 2, the number of T-cell epitope

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sites recognized by each patient was 8.7 ± 3.3 on average and ranged from $2 \leq 13$ epitopes. Since Cry j 1 is composed of 353 amino acids (International patent application published in Japan No. Hei 8-502163) and Cry j 2 is composed of 379 amino acids (JP-A No. Hei 8-47392), the above results mean that about 2.3 to 2.8 T-cell epitope sites exist per 100 amino acid residues. Each patient has different HLA class II types and therefore recognizes different T-cell epitopes depending on the HLA class II types. An epitope map was prepared by marking T-cell epitope sites on the Cry j 1 or Cry j 2 molecule T-cell epitope sites recognized by each patient on the Cry j 1 or Cry j 2 molecule. The results are shown in Figs. 1 and 2.

Example 6 Identification of T-cell epitopes recognizing T cell clones

Among 18 patients suffering from a cryptomeria pollen allergy, 2 patients recognizing antigen peptides p211-225 and p106-120 of Cry j 1 [patient B (hereinafter referred to as PB), and patient J (hereinafter referred to as PJ)], 3 patients recognizing antigen peptides p66-80, p186-200, p236-250, and p341-355 of Cry j 2 [PB, patient C (hereinafter referred to as PC), and patient R (hereinafter referred to as PR)] were selected. T-cell clones recognizing Cry j 1 or Cry j 2 were established by stimulating peripheral blood lymphocytes of these cryptomeria pollen allergy patients by Cry j 1 or Cry j 2. HLA class I and II types of the four patients are as follows: PB: A2/24 - B39/55 - Cw7/w3 - DRB1*1501/0901 - DRB4*0101 - DRB5*0101, DQA1*0102/0301 - DQB1*0602/0303 - DPA1*0101/0101 - DPB1*0501/0201; PJ: A24/ - - B61/51 - Cw3/ - - DRB1*1501/0802 - DRB5*0101, DQA1*0102/0401 - DQB1*0602/0402 - DPA1*-/- - -

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DPB1*0501/0402; PC: A-2/2 - B54/51 - Cw1/-, DRB1*0405/1501 - DRB4*0101
- DRB5*0101 - DQA1*0301/0102 - DQB1*0401/0602 - DPA1*0202/0202 -
DPB1*0201/0501; PR: A-11/ - - B60/35 - Cw7/w3 - DRB1*0901/1501 -
DRB4*0101 - DRB5*0101 - DQA1*0301/0102 - DQB1*0303/0602 -
DPA1*01/0202 - DPB1*0201/0201.

35 and 14 types of T-cell clones specifically recognizing Cry
j 1 were established from the peripheral blood lymphocytes derived
from PB and from PJ, respectively. Similarly, 31, 10, and 17 types
of T-cell clones specifically recognizing Cry j 2 were established
from the peripheral blood lymphocytes derived from PB, PC, and PR
respectively. All of these T-cell clones were CD3⁺, CD4⁺, CD8⁻, TCR
 $\alpha\beta$ ⁺, TCR $\gamma\delta$ ⁻, therefore, the restriction cells were found to be HLA
class II molecules. Self-derived 5×10^4 B-cell lines treated with
mitomycin C, $2 \mu\text{M}$ of the overlapping peptides, and 2×10^4 T-cell clones
were cultured in RPMI-1640 medium supplemented with 0.2 ml of 15%
serum on a 96-well microplate for 2 days. After $0.5 \mu\text{Ci}$ of [³H]
thymidine was added, the cells were further cultured for 18 hours.
The cells were collected in a glass filter by a cell harvester and
uptake of [³H]thymidine was measured using a liquid scintillation
counter. T-cell epitopes recognized by each T-cell clone were
identified by the above manipulation. Sixty-nine percent (34/49) of
T-cell clones recognizing Cry j 1 proliferated in response to
stimulation by the peptide containing T-cell epitopes and the antigen
peptides were identified. Similarly, antigen peptides were
identified among 69% (40/58) of T cell clones recognizing Cry j 2.
T-cell clones specifically recognizing Cry j 1 recognized peptides

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p16-30, p61-75, p91-105, p106-120, p146-160, p151-165, p191-205, p211-225, p251-265, p326-340, and p331-346. T-cell clones specifically recognizing Cry j 2 recognized peptides p16-30, p21-35, p36-50, p66-80, p76-90, p81-95, p151-165, p181-195, p186-200, p236-250, p321-335, p326-340, p336-350, p341-355, and p346-360. The results are summarized in Figures 1 and 2 (the histograms in the center).

Example 7 Identification of HLA class II restriction molecules in the locus level

HLA class II restriction molecules were identified in the locus level by adding monoclonal antibodies specifically reacting with HLA-class II-DR, HLA-class II-DQ, or HLA-class II-DP to the proliferation response system of T-cell clones established in Example 4 so as to inhibit T cell proliferation response.

Self-derived 2×10^4 B-cell lines treated with mitomycin C, $2 \mu\text{M}$ of the overlapping peptides, $3 \mu\text{g/ml}$ of anti-DR, DQ, or DP monoclonal antibody (Becton/Dickinson), and 2×10^4 T-cell clones were cultured in RPMI-1640 medium with 0.2 ml of 15% serum for 2 days. After $0.5 \mu\text{Ci}$ of [^3H]thymidine was added, culturing was performed for further 18 hours. The cells were collected in a glass filter using a cell harvester, and uptake of [^3H]thymidine was measured using a liquid scintillation counter.

Example 8 Identification of restriction molecules of each type of HLA class II molecules

Restriction molecules of each HLA class II type of T-cell clones whose restriction molecules were identified in the locus level can

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be identified by using, as antigen-presenting cells, mouse L-cells transformed with the DR gene and B-cell lines having the same haplotype as DQ or DP.

5 x 10⁴ of the above mouse L-cells or haplotype matching B-cell lines, treated with mitomycin C, 2 μ M of overlapping peptides, 3 μ g/ml of anti-DR, DQ, or DP monoclonal antibody (Becton/Dickinson), and 2 x 10⁴ T-cell clones were cultured in RPMI-1640 medium with 0.2 ml of 15% serum for 2 days. After 0.5 μ Ci of [³H]thymidine was added, culturing was further continued for 18 hours. The cells were collected in a glass filter using a cell harvester, and uptake of [³H]thymidine was measured using a liquid scintillation counter. Restriction cells can be identified when proliferation response of T-cell clones are observed. The results of the analysis are shown in Figures 3 and 4.

Example 9 Identification of Th types for T cell clones

Th2 cells are presumably involved in onset of allergy. It has not been revealed yet as to whether differentiation of T cells to Th1 or Th2 cells in response to antigenic stimulation is controlled by the specific epitope peptides or the HLA-class II locus. If Th2 cells are primarily induced after stimulation by the peptides for selecting antigen peptides, cryptomeria pollen allergy would worsen due to administration of the peptides. To examine the above hypothesis, Th types for the T-cell clones prepared in Example 4 were determined by stimulating the clones by the epitope peptides recognized by the T cells, and measuring production of IL-2, IL-4, and IF- γ .

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Specifically, self-derived 1×10^5 B-cell lines treated with mitomycin C, $2 \mu\text{M}$ of epitope peptides, and 5×10^5 T-cell clones were cultured in RPMI-1640 medium with 1 ml of 10% human serum for 24 hours, then the supernatant was obtained by centrifugation. IL-2, IL-4, and IFN γ in the supernatant were determined using the commercially available ELISA kit [IL-2 (R&D), IL-4 (Medojenics), and IFN γ (Otsuka Assay Laboratory)].

Sub C34 Figures 3 and 4 show the production of IL-2, IL-4, and IFN γ and Th types of each clone. The number of T-cell clones recognizing Cry j 1 are 12 in Th2 cells, 1 in Th1 cells, 16 in Th0 cell, thus the number of Th2 was larger than that of Th1. In contrast, the number of T-cell clones recognizing Cry j 2 are 10 in Th2 cells, 8 in Th1 cells, 8 in Th0 cells, thus the number of Th1 was almost the same as that of Th2. Comparing T-cell epitopes recognizing each T cell clone, the restriction molecules, and Th types, each T-cell clone was different in Th2, Th1, and Th0 types. For several T cell clones recognizing the same epitopes and same antigen-presenting molecules, both Th2 and Th1 cells were identified. These findings indicate that differentiation of T cells to Th2, Th1, or Th0 cells after stimulation by Cry j 1 or Cry j 2 is not determined by the combination of specific T-cell epitopes or specific restriction molecules. In other words, any peptide containing T-cell epitope sites can stimulate T cells and can be selected as peptides for use as a peptide-based immunotherapeutic agent.

Example 10 Identification of T-cell epitopes in CB6F1 mouse

Sub C34 Eight-week-old male CB6F1 mice were immunized with 10 μg of

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recombinant Cry j 2 (rCry j 2) together with an adjuvant (Imject Alum, PIERCE) three times every two weeks (ip). One week after the last immunization, splenocytes were prepared from three mice and combined. 5×10^6 splenocytes were cultured, together with $0.115 \mu\text{M}$ of 74 types of the overlapping peptides consisting of 15 amino acid residues, in 0.2 ml of RPMI medium (10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 $\mu\text{g/ml}$ streptomycin) in each well of a 96-well plate (Falcon). As the control, the responses to PBS, 50 $\mu\text{g/ml}$ of Cry j 1, 0.3 $\mu\text{g/ml}$ of rCry j 2 were assessed. Each reagent was inoculated in three wells and cells were cultured at 37°C under 5% CO_2 for three days. Pulse labeling was performed with 0.5 $\mu\text{Ci/well}$ of [^3H]thymidine for the last 6 hours and the cells were collected in a glass filter using a cell harvester (Inotech, Bertold Japan). After the cells were dried, uptake of [^3H]thymidine into the cells was measured with a liquid scintillation counter (TRI-CARB 4530, Packard Japan)

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CB6F1 mice immunized with rCry j 2 showed a strong response to rCry j 2 antigen, but did not respond to another cryptomeria pollen major allergen Cry j 1, indicating that this system was antigen-specific reaction. CB6F1 mice immunized with rCry j 2 showed the remarkable responses to p66-80 (SEQ ID NO: 14) and p236-250 (SEQ ID NO: 48) among tested 90 kinds of overlapping peptides. These results indicated that p66-80 and p236-250 peptides involved in the antigen presentation as a major T-cell epitope in CB6F1 mice. In a human, p66-180 (SEQ ID NO: 14) and p236-250 (SEQ ID NO: 48) are also major T cell epitope peptides. Thus, a CB6F1 mouse can be a useful model animal to evaluate the effectiveness of peptide compositions to be

used in peptide-based immunotherapy for cryptomeria pollen allergy.

Example 11 In vivo immunoreaction of antigen peptide p66-80 (SEQ ID NO: 14)

Three mg of p66-80 peptide (SEQ ID NO: 14) dissolved in physiological saline was subcutaneously administered to an eight-week-old male mouse twice at an interval of 5 days. Similarly, the same volume (100 μ l) of physiological saline was administered to mice of the control group. Each of the peptide-administered group and the control group had eight mice. Five days after the second peptide administration, 50 μ g of rCry j 2 mixed with an adjuvant, Imject Alum, was subcutaneously administered to all mice for immunization. One week after the immunization, splenocytes were prepared from each mouse. 5×10^6 splenocytes were cultured together with 3 μ g/ml of rCry j 2 in 0.2 ml of RPMI medium (10% FCS, 2mM L-glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin) in each well of a 96-well plate (Falcon). As the control, the cells were cultured in the same medium containing no rCry j 2. Uptake of [3 H]thymidine was measured as described in Example 10.

When p66-80 (SEQ ID NO: 14) was subcutaneously administered to CB6F1 mice before antigen stimulation by rCry j 2, immune response of the T cells was significantly inhibited compared to the physiological saline-administered group ($p < 0.01$). This result indicated that in the mouse model p66-80 (SEQ ID NO: 14) system showed a preventive effect in peptide-based immunotherapy for treating cryptomeria pollen allergy.

Example 12 In vivo immune response to antigen peptide p236-250 (SEQ

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Three mg of p236-250 peptide (SEQ ID NO: 48) dissolved in physiological saline was subcutaneously administered to a six-week-old male mouse twice at an interval of 5 days. As a control, the same volume (200 μ l) of physiological saline was administered to mice in the same manner as above. Each of the peptide-administered group and the control group had eight mice. Five days after the second peptide administration, 50 μ g of rCry j 2 mixed with adjuvant Imject Alum was subcutaneously administered to all mice. One week after the immunization, splenocytes were prepared from each mouse. 5×10^6 splenocytes were cultured together with 3 μ g/ml of rCry j2 in 0.2 ml of RPMI medium (10% FCS, 2mM L-glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin) in each well of a 96-well plate (Falcon). As a control, the cells were cultured in the same medium containing no rCry j 2. Uptake of [3 H]thymidine was measured as described in Example 10.

When p236-250 (SEQ ID NO: 48) was subcutaneously administered to CB6F1 mice before antigenic stimulation by rCry j 2, immune response of the T cells was significantly inhibited compared to the physiological saline-administered group ($p < 0.05$). This result indicated that in the mouse model system p236-250 (SEQ ID NO: 48) showed a preventive effect in peptide-based immunotherapy for treating cryptomeria pollen allergy (Figure 6).

The above results revealed that the conventional hyposensitization in humans using a cryptomeria pollen extract was based on the mechanism mediated by T-cell epitopes.

